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Electrophoresis

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Introduction

It is somewhat arbitrary to outline electrophoretic applications that are used in a routine clinical laboratory because they are highly dependent on the specificity of each application. Nowadays, a multitude of electrophoretic methods are routinely used in various clinical laboratories, according to the specific research being developed. More and more sophisticated methods are needed to resolve specific clinical problems, the reason why specialization of laboratories is mandatory in order to provide accurate results at the lowest possible cost. In addition, quality control is of major importance. Therefore, automation is progressively introduced in all the steps involved in the analytical process, and only a few manual methods will survive in the future. However, highly sophisticated electrophoretic techniques should be maintained and developed in a limited number of specialized laboratories, in order to resolve the different problems that are encountered in clinical medicine. Here, we present selected examples of electrophoretic techniques that are employed in the clinical laboratory.

Serum Protein Electrophoresis

Electrophoretic techniques for the separation of human serum proteins have been used for fifty years. Resolution has been improved by the use of support media such as paper, starch gel, cellulose acetate, agarose, and polyacrylamide gels, which have rendered electrophoretic methods very popular in the diagnostic area. However, many of those methods have remained labour intensive, being difficult to automate. Serum protein electrophoresis is widely used in clinical laboratories, especially for the evaluation of changes in proteins associated with inflammation, liver or kidney diseases as well as for the detection and identification of paraproteins. Traditional clinical electrophoretic procedures are manual methods that use agarose gels or cellulose acetate membranes as the separation bed. Quantitation of the five major serum fractions is done by densitometric scanning of the gel or the membrane. Clinical interpretation is based on the alteration of the content of one or more of the five fractions. Agarose, as supporting medium for protein electrophoresis, has been reported to give better resolution as well as to allow better detection of paraproteins than is cellulose acetate. Semiautomated agarose electrophoresis and immunofixation can be performed with various commercially available systems. No differences between manual and semiautomated methods have been seen with respect to paraprotein identification.

Over the last few years, capillary zone electrophoresis (CZE) has emerged as a powerful new tool for rapid separation of various biopolymers, including proteins. Separation by this technique depends on the electrophoretic mobility of the analyte and the electroosmotic flow of the bulk solution, and can be easily automated. Direct quantitation of proteins via peptide bonds is possible using UV detection at 214 nm. Separation patterns obtained by CZE are similar to those obtained after densitometric scanning of cellulose acetate membrane electrophoresis or agarose gel electrophoresis. Recently, dedicated automated systems for the routine analysis of human serum proteins in clinical laboratories have become commercially available. High sample throughput is attained due to the presence of several fused-silica capillaries, which allow the simultaneous analysis of different samples. Several studies have clearly shown that the electrophoretic patterns and the clinical information obtained by CZE are comparable with the data obtained by classical methods. In addition, the method is also suited to detect monoclonal gammopathies. Multicapillary instruments have been

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designed for automation of both routine serum protein electrophoresis and for monoclonal component typing by subtraction (immunofixation electrophoresis – immunosubtraction). In this latter technique, CZE is performed on the supernatant of serum samples that have reacted with Sepharose beads coated with an immunospecific binder (IgG, IgA, IgM, κ and λ). The low detection limit of the technique (0.5 g L^{-1} for IgG, 0.75 g L^{-1} for IgA and IgM) contributes to the ability of CZE to detect small monoclonal gammopathies.

Lipoprotein Analysis

The cardiac risk profile that is routinely measured in the clinical laboratory is an important indicator of susceptibility to the development of atherosclerosis. There is clearly a need to develop a more comprehensive cardiovascular risk profile that includes more factors than cholesterol and triglycerides. Triglycerides and cholesterol are transported in blood in association with lipoproteins, that can be separated by ultracentrifugation into four main classes according to their density: chylomicrons, very low-density lipoproteins (VLDL), low-density-lipoproteins (LDL), and high-density lipoproteins (HDL). The current lipid profile measured focuses on the lipid components of lipoproteins. Because proteins associated with lipoproteins, apoproteins, play a central role in lipid homeostasis, important research efforts have been directed to the study of apoproteins.

Free-flow isotachophoresis (ITP) and capillary ITP (cITP) has been utilized for many years to study plasma lipoproteins. The discriminating principle is based on the net charge of lipoproteins. cITP is a high-resolution electrophoretic technique, by which ionic sample components are separated according their net electric charge and without molecular sieve effects. With cITP, lipoprotein analysis can be performed directly from whole serum and offers the potential of a reliable and automated quantitation of lipoprotein subpopulations. Sample pretreatment is negligible and only few nanolitres of sample are necessary for the analysis. LDL are a heterogeneous population of particles that varies in size, density, composition, and electric charge. One significant aspect of this variability, relevant to atherosclerosis, is the phenotypic pattern of LDL density and size distribution among individuals with and without coronary artery disease. Using nondenaturing gradient gel electrophoresis, various LDL subgroups have been identified on the basis of size. Individuals with lipoprotein profiles enriched in small dense LDL were found to be predisposed to coronary artery disease. Higher plasma levels of LDL have been found in patients with acute myocardial infarction, unstable angina, and thrombogenic carotid atherosclerosis. The method of study of LDL, which uses ion exchange chromotography of LDL isolated by ultracentrifugation, requires particular care to avoid artefacts due to *in vitro* auto-oxidation of the LDL and is time consuming. HDL are a family of protein–lipid complexes that play a central role in cholesterol transport. HDL collectively contain apolipoproteins (apo) A-I and A-II as major protein components, together with apoC, E, and A-IV. Changes in HDL composition occur during normal metabolism, as the result of a particular genetic profile, or as a consequence of disease. The protein composition of lipoproteins can be analysed by chromatography, electrophoresis, or immunoassay. SDS-PAGE resolves proteins according to molecular size, while charge separation can be achieved by zone electrophoresis or isoelectric focusing. Quantification of protein bands after electrophoresis is achieved by immunoblotting or by staining with Coomassie Blue. However, due to differences in the chromogenicity of different apolipoproteins, these staining methods are only semiquantitative. Immunoassays offer good sensitivity and precision, but results can vary with different antisera. Furthermore, immunoassays do not distinguish between different apo isoforms. The availability of high performance capillary electrophoresis systems has created the possibility of applying this technology to lipoprotein analysis. Protein separation is performed at high field strengths in micropore capillaries, with direct monitoring by online UV detection. The separation is analogous to that achieved with slab gel systems, except that no support media are used, electrophoresis being carried out in a free solution, and results are obtained in minutes rather than hours. Separations based on differences in molecular size are performed in a SDS-containing UV transparent polymer network. This mode, usually termed 'capillary SDS gel electrophoresis' (though it actually uses a non-gel matrix), has been found to give similar results to those obtained with SDS slab gel electrophoresis.

Determination of Serum Protein Phenotypes and Microheterogeneities

The determination of the phenotype of particular serum proteins may have clinical relevance. Hereditary deficiencies of the proteinase inhibitor α_1-antitrypsin, one of the most common inborn metabolic errors in Europeans, lead to pulmonary emphysemia in young adults and to liver cirrhosis in children. Many distinct subtypes have been identified using isoelectric focusing, the most common normal phenotype being MM, whereas the major deficient

phenotypes are termed MS, MZ, SS, SZ and ZZ. The last form, ZZ, is associated with low concentration of the protein in plasma and with severe clinical manifestations. Isoelectric focusing in immobilized pH gradients represents a major improvement, and the method can be utilized for subtyping many different proteins with high reproducibility.

The microheterogeneity of serum glycoproteins in patients with chronic alcohol abuse as well as in patients with carbohydrate-deficient glycoprotein syndrome can be globally studied by two-dimensional electrophoresis, whereas SDS-PAGE and Western blotting allows detailed comparison and characterization of specific proteins.

Diagnosis of Haemoglobinopathies and Haemoglobin A1c

Disorders of haemoglobin synthesis such as sickle cell disease, β- and α-thalassaemias or haemoglobin variants – grouped under the term 'haemoglobinopathies' – are frequently observed, and up to 45% of the newborns from regions at risk present an abnormal haemoglobin. Therefore, it is of importance to have cost-effective methods to screen large populations. Isoelectric focusing has been used for many years, and when performed correctly, produces excellent haemoglobin separation, with very little band overlap when bands are measured to 0.1 mm against controls. It has been shown that high-power liquid chromatography also allows accurate diagnosis of the haemoglobinopathies, and that both approaches can be used for a universal screening. Another interesting approach to study haemoglobin variants is capillary isoelectric focusing. The technique allows high-efficiency separation and precise quantitation of haemoglobin variants over a wide range of concentrations.

Haemoglobin A1c (Hb A1c) is the analyte of choice for monitoring metabolic control in patient with diabetes mellitus. Hb A1c can be measured using capillary electrophoresis, without apparent interference by other haemoglobin variants such as Hb F, Hb S or Hb C.

Genotyping of Proteins

Many complex biological questions, encountered in all fields of medicine, have been solved using DNA-based technologies. It is now possible to make many different prenatal diagnoses using genetic testing with one technique or a combination of the three basic molecular testing techniques – nucleotide sequencing, restriction fragment length polymorphism mapping and molecular DNA analysis using the polymerase chain reaction (PCR) – routinely used to assess gene mutations. Apolipoprotein E (apoE) is a secreted glycoprotein with a molecular mass of 35 kDa which is synthesized primarily in the liver and brain. ApoE plays a critical role in lipid metabolism through its function of redistributing lipids amongst the cells of various organs. It is a constituent of VLDL and of a subclass of HDL. The *APOE* gene is polymorphic and its three common alleles $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ code for isoforms E2, E3 and E4, respectively. The iso-forms E3 and E4 differ from E2 by single arginine (R) substitution at one or both cysteine (C) at position 130 and 176 of the apoE amino acid sequence (accession number P02649; SwissProt database). Compared with allele $\varepsilon 3$, reduced LDL cholestrol (LDL-c) and apoB levels frequently accompany the $\varepsilon 2$ allele, higher LDL-c levels occurring with the $\varepsilon 4$ allele, and both $\varepsilon 2$ and $\varepsilon 4$ alleles are associated with hypertriglyceridaemia. In addition to the dyslipidaemic tendency, the relative odds for prevalent coronary heart disease are found to be increased with the $\varepsilon 4$ allele. A compelling association between risk of Alzheimer's disease in both late-onset familial Alzhemer's disease and sporadic Alzheimer's disease with the $\varepsilon 4$ allele has been also demonstrated. *APOE* genotyping is usually determined by a blood test using DNA isolated from leukocytes, embedded tissues, Guthrie spots, buccal epithelial cells and restriction isotyping. The method is based on the existence of a GCGC recognition site for the endonuclease *Hha*I overlapping the coding sequences present for amino acids 130 and 170 in the $\varepsilon 4$ allele, absent in position 130 in the $\varepsilon 3$ allele, and absent in both position 130 and 170 in the $\varepsilon 2$ allele. A 244-bp sequence including the two polymorphic sites is amplified by using PCR and is digested by *Hha*I (**Figure 1**). The resulting fragments are separated by electrophoresis on polyacrylamide gels. After electrophoresis, the gels are either treated with ethidium bromide or are silver stained. The method cannot detect rare *APOE* variants unless nucleotide substitution alter the *Hha*I-restriction sites within the PCR-amplified region. In that case the rare variants are identified only by using DNA sequencing. PCR and restriction isotyping for *APOE* genotyping must be preferred to isoelectric focusing (IEF). Because the post-translational modifications of apoE such as glycosylation or desialylation alter the electric charges of the isoforms, misclassification of genotypes currently occurs when using IEF.

Small Molecules (Drugs, Steroids) Monitoring

Drugs, licit or illicit, play a pivotal role in almost all aspects of life. Monitoring of drugs is of major importance for regulatory authorities, in clinical

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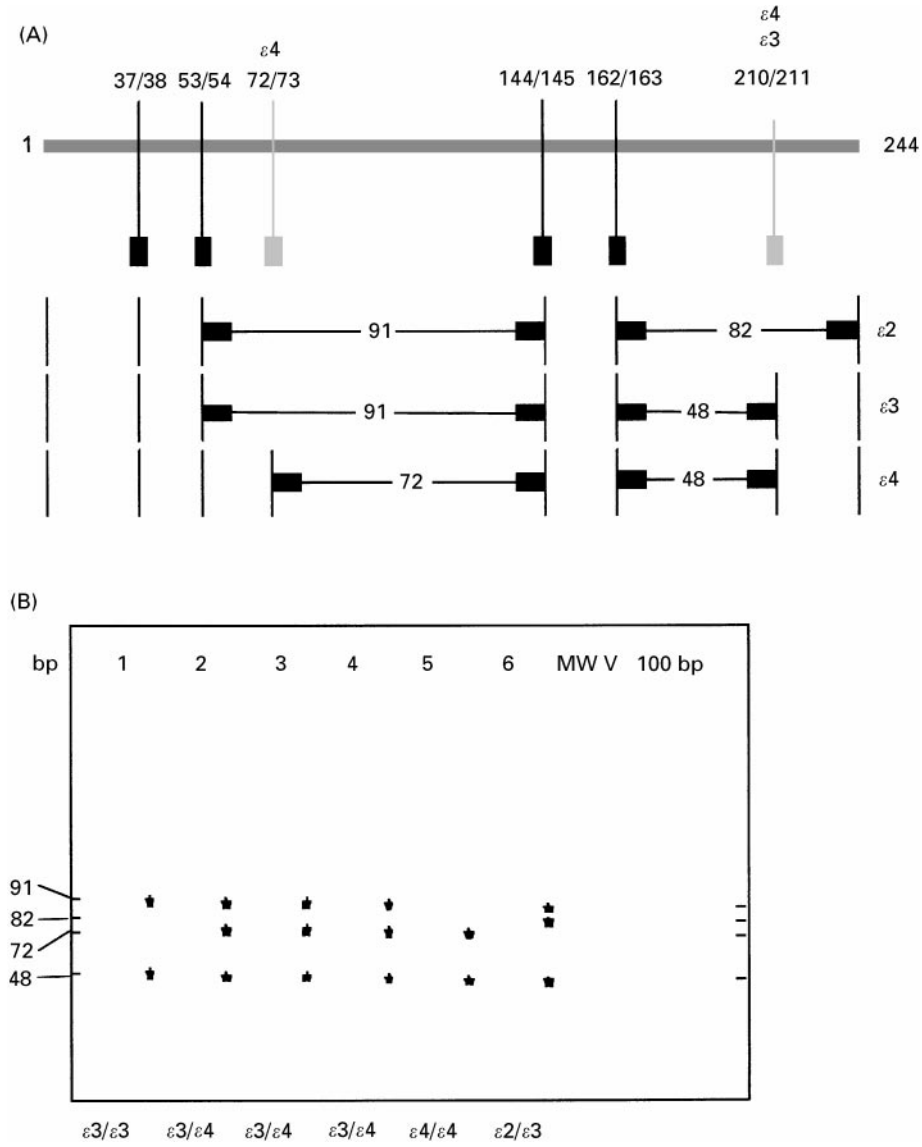

Figure 1 Application of PCR to the analysis of apolipoprotein E. (A) Map of the PCR-amplified region encoding common *APOE* isoforms and location of *Hha*I cleavage sites. The distance in basepairs between the *Hha*I sites that distinguish isoforms are shown for each genotype. (B) Silver-stained PAGE after the electrophoretic separation of *Hha*I fragments from different patients.

settings, in forensic science, for drug testing at the workplace, and in the pharmaceutical industry. With the advent of fused-silica capillary instrumentation, drug analysis by capillary electrophoresis has made important progress. Capillary electrophoresis of drugs, as well as their metabolites in almost all biological samples, has been shown to provide high-quality data that can be used for diagnostic drug monitoring.

Corticosteroid hormones are usually analysed by immunological techniques or HPLC. Immunoassays are prone to interference from various compounds, a disadvantage not observed with HPLC. However, the latter technique is hampered by the large sample and eluent volumes needed. Corticosteroid hormones such as cortisone, cortisol, progesterone and oestrogen can be studied using mixed micellar electrokinetic capillary chromatography (MEKC), using borate buffer and SDS. The separation is rapid and quantitation is efficient.

Cerebrospinal Fluid Analysis

Analysis of cerebrospinal fluid (CSF) proteins using electrophoretic techniques is particularly useful in the diagnosis and management of neurological diseases particularly in the detection of immune response within the central nervous system (CNS). In healthy individuals, all CSF immunoglobulins are derived from plasma. However, in most of the inflammatory conditions within the CNS, activated B-lymphocytes

secrete their immunoglobulins directly into the brain extracellular space. This local immune response occurs typically in demyelinating disorders such as multiple sclerosis, in chronic CNS infections as well as in autoimmune disorders with neurological involvement. An intrathecal synthesis of immunoglobulins can be detected either by quantitative methods and the results are expressed using various indexes or by qualitative methods such as IEF and Western blotting. Because the locally synthesized immunoglobulins are the product of a limited number of clones, they currently produce an oligoclonal pattern superposed to the plasma pattern (**Figure 2**). IEF is now considered as a more sensitive test than the quantitative index for the detection of an intrathecal immune response. The diagnostic sensitivity and specificity of both quantitative and qualitative tests has been evaluated in samples from 1007 patients with neurological diseases. It was found that IEF has a sensitivity of 95% for multiple sclerosis against 67% for the quantitative tests and a specificity with a false-positive rate of 0% versus 3.5% for the quantitative tests.

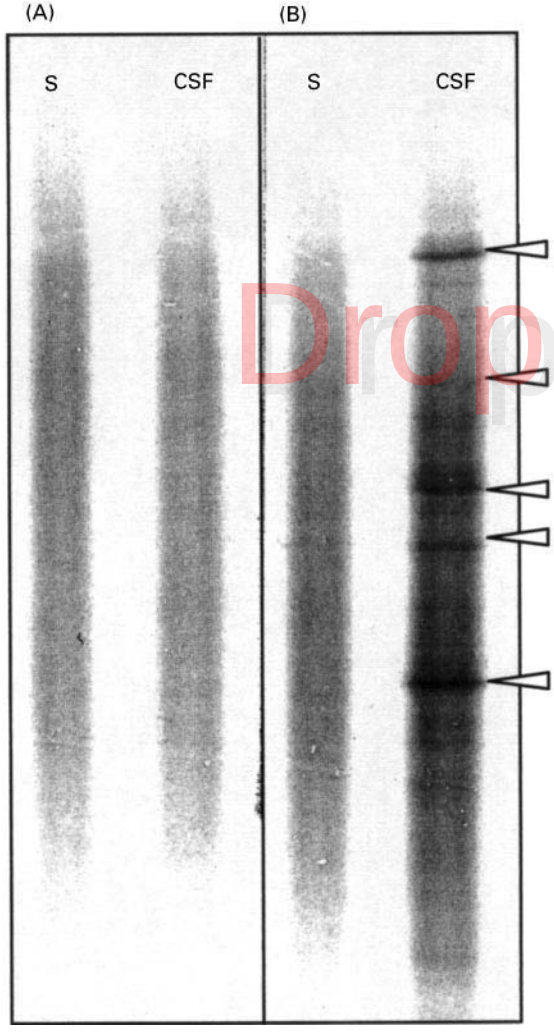

Figure 2 Analysis of the immunoglobulins of the cerebrospinal fluid. Typical IgG patterns of sera (S) and of cerebrospinal fluid (CSF) separated by isoelectric focusing and revealed by Western blot. (A) Normal control. (B) Patient with multiple sclerosis. The arrow heads indicate the oligoclonol bands found only in the CSF.

The diagnosis and the treatment of the discharge of CSF (liquorrhea) from the subarachnoid space into the nasal or aural mucosa (CSF rhinorrhea and otorrhea) is a critical clinical problem. The most common cause of liquorrhea is traumatic fracture: in a large series of pediatric patients with temporal bone fractures, liquorrhea was noted in 25% of the patients. Other causes are neurosurgical procedures, intra- and extracranial tumours, as well as primary CSF rhinorrhea through congenital bony dehiscences. The diagnosis of liquorrhea is important in view of developing a potentially fatal meaningitis. The use of prophylactic antibiotics does not appear to be benefical in reducing the incidence of post-traumatic meningitis. Surgical intervention is therefore necessary. Detection of β_2-transferrin is now considered as the most reliable test in the identification of CSF rhinorrhea and otorrhea. β_2-transferrin is a transferrin isoform which is locally synthesized in the central nervous system. This isoform is characterized by the presence of truncated *N*-glycans devoid of terminal galactose and sialic acid. The serum transferrin and β_2-transferrin isoforms found in CSF are separated by native electrophoresis using agarose and detected by immunoblotting. Because there is no gold standard for the diagnosis of liquorrhea, the sensitivity and the specificity of the test are unknown.

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Urine Analysis

Electrophoretic methods have been widely used for the clinical analysis of the proteins contained in urine (proteinuria). Normally, the renal glomeruli restrict filtration of plasma proteins, and only traces are present in the urine. In several renal diseases and particularly in patients with glomerulonephritis, nephrosis, glomerulosclerosis and IgA nephropathy, large amounts of proteins may be present in the urine. This is the reason why so many different electrophoretic techniques, including SDS-PAGE, isoelectric focusing, and high-resolution two-dimensional polyacrylamide gel electrophoresis have been applied over the years to study in detail the protein composition of the urine of patients with kidney diseases.

The Diagnosis of Infectious Diseases by Western Blotting

Despite the analytical power of gel electrophoresis, direct specific identification of separated components

by ligands such as antibodies, lectin or enzymes was generally hindered by the pore size of the gel matrix. Thus the development of protein blotting techniques that enabled the separate components to be transferred from gels onto membranes where they were bound and available for participation in a range of reactions was of enormous practical significance. Basically, with these techniques, viral proteins, either from culture fluid or obtained by recombinant molecular technologies, are separated by SDS-PAGE, then blotted from the polyacrylamide gel matrix onto membranes by an electrophoretic transfer, and finally identified using enzyme immunoassay. The Western blot method has been used in key epidemiological studies that reported the unambiguous association of human immunodeficiency virus (HIV) with AIDS, and is still an essential tool for confirming the presence of antibodies to HIV. Western blot has been used as the 'gold standard' confirmatory test for specimens found to be reactive in screening assays. Protein blotting can be applied to a large variety of infectious diseases, to study allergens in patients with allergy as well as to identify autoantigens in patients suffering from autoimmune disorders.

The transmissible spongiform encephalopathies or prion diseases constitute a group of progressive and fatal neurodegenerative diseases that affect both human and animals. The diseases affecting humans include Creutzfeldt–Jakob disease (CJD) for which three main forms have been recognized: inherited, sporadic, and acquired (iatrogenic). Patients with CJD present a progressive dementia with myoclonus usually associated with the presence of sharp-wave complexes on their electroencephalogram. The gold standard for a definitive diagnosis of CJD require a histological examination of the brain and immunostaining for the protease-resistant prion protein (PrP^{res}). Several CSF proteins such as neurone-specific enolase, S-100b, CK-BB, ubiquitin and protein 14-3-3 have been proposed as potential pre-mortem markers for CJD. Amongst them, only protein 14-3-3 proved useful diagnostically. The differential expression of CSF proteins in patients with CJD has been evaluated by using two-dimensional electrophoresis, and two protein spots with CJD has been evaluated by using two-dimensional electrophoresis, and two protein spots (proteins 130 and 131) were identified. *N*-terminal sequencing of protein 130 matches the sequence of 14-3-3β, a brain-specific protein of 28 kDa which is believed to have a regulatory function in monoamine biosynthesis. A Western blot assay has been developed, using a commercially available antibody directed against 14-3-3β (Santa Cruz Biotechnology) which cross-reacts with protein 130 and 131 (**Figure 3**). It was found that the 14-3-3 immunoassay had an overall sensitivity of 96% (68 true-positive results and 3 false-negative results) and an overall specificity of 88% (164 true-negative and 22 false-positive results). Interestingly, the specificity of the immunoassay amongst all patients with dementia was 96% (90 true-negative and 4 false-positive results). It was also observed that the immunoassay may become positive early in the course of CJD. However the immunoassay does not allow a quantitation of the 14-3-3 proteins and needs further validation with larger numbers of patients.

Identification of Microorganisms

Pulsed-field gel electrophoresis (PFGE) is a method widely used to separate fragments of DNA as long as several million bases by subjecting the gel to an electrical current alternately delivered from two angles in timed intervals, which minimizes diffusion of large molecules. PFGE has many important clinical applications, particularly in the field of infectious diseases. PFGE allows typing bacteria such as group A streptococci, *Staphylococcus aureus*, *Escherichia coli* or *Salmonella enterica*. PFGE can be particularly useful for assisting epidemiological investigations of illnesses caused by a common-source of pathogen such as *Escherichia coli* O157 : H7 in food poisoning or to demonstrate that recurrent episodes of staphylococcal bacteraemia are primarily related to relapses rather than to new infections. The technique is commonly known as DNA fingerprinting.

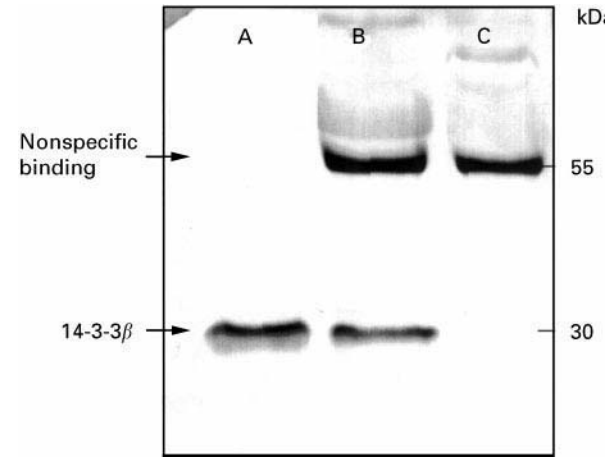

Figure 3 Application of Western blot to the diagnosis of Creutzfeldt–Jakob disease. Immunoblot by using anti-14-3-3β after SDS-PAGE separation of (A) human brain proteins, (B) cerebrospinal fluid proteins from a patient with Creutzfeldt–Jakob disease and (C) normal control cerebrospinal fluid. Note that anti-14-3-3β immunoglobulins bound nonspecifically to a cerebrospinal fluid protein with a molecular mass close to 55 kDa.

Summary

Electrophoretic techniques will certainly continue to be applied in the clinical laboratory for many years to come. However, only highly reproducible methods such as those based on capillary electrophoresis will survive on the condition that they can be applied to resolve heterogeneous clinical problems and that they can be fully automated with electronic handling of the data. The evolution of electrophoretic techniques achieved over the last few years has allowed a revolution in medical science. However, in terms of manual operations, the good old time of 'blue fingers' has probably finished.

See Colour Plate 68.

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